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A SEROPREVALENCE STUDY OF DENGUE VIRUS INFECTION AMONG CLINICALLY SUSPECTED PEDIATRIC PATIENTS IN CHENNAI, TAMILNADU

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ABSTRACT

Dengue is a fast emerging pandemic-prone viral disease in many parts of the world. The objective of the study was to report the seroprevalence of dengue virus infection among clinically suspected pediatric patients reporting to children's hospital from August 2011 to October 2011 in Chennai, Tamilnadu, India. This retrospective study was analyzed from 100 dengue patients reporting to hospital. Of these, 94 serum samples were subjected to lgM ELISA and confirmed by RT-PCR and also to find out the prevailing serotype. Positive cases were seen as a definite dengue infection, while those that were only lgM positive were considered as 'Possible' dengue. Of the 94 suspected serum samples 37 (39.4%) were positive for lgM ELISA. An aliquot of all the suspected samples 12 (12%) which were positive and all the confirmed dengue virus were subjected for serotype confirmation which is highly significant (P<0.000). Among the RT-PCR results, 10 cases were detected for dengue 1 which is predominant and 2 cases were detected as dengue 3 serotype. Referable to the cost effectiveness, one predominant serotype was sequenced and published in NCBl and also propagated for further work. This study proposed the predicted outbreak thus emphasizes the need for continuous sero epidemiological surveillance for the timely formulation and implementation of effective dengue control programs.

KEYWORDS: Seroprevalence, Dengue Fever, lgM ELISA, RT-PCR, Childrens

INTRODUCTION

The worldwide prevalence of dengue has grown dramatically in recent decades. Population based studies suggest that asymptomatic infections are the main outcome of dengue virus exposure. However, whenever DHF occurs, it is associated with high morbidity and mortality Martina et al. (2009). According to World Health Organization (WHO) estimates, over 100 countries and approximately 40% of the world's population (nearly 2.5 billion people) are threatened by DENV. The exponential growth of dengue virus cases i.e augmented incidence of dengue has been ascribed to increased air-travel, increased urbanization, amplified mosquito population due to deterioration in the public health infrastructure and shifting climatic conditions Kyle and Harris (2008). Presently, about 100 million DENV infections occur worldwide every year, and the WHO has classified dengue as a major international public health concern Gubler (2002). Globalization has also increased the spread of viruses and mosquito (vectors) hence, epidemics and outbreaks have occurred with increased frequency in recent years Wilder and Shwartz (2005).

In India, the disease is prevalent and all four serotypes are known to be circulating either singly or in combination among the serotypes resulting in several outbreaks over the years and it is to be noted that the disease is much more severe in children than adults. lgM antibodies of dengue virus appear within 3 days after infection, which may remain in circulation for 10 days to 2 months, whereas the lgG antibodies of dengue virus will appear after 1 week and remain lifelong in circulation. As effective control and preventive programs for dengue infection are based upon improved surveillance data, this study was done to report the seroprevalence of dengue virus infection in Chennai. Infection with any

one of the serotypes can cause severe clinical manifestations, Moreover, natural infection with any of the serotypes can only provide long-term homotypic immunity, which leads to a higher risk for DHF/DSS during secondary infections with a heterogenous serotype (Kliks 1989).

Dengue and Dengue hemorrhagic fever, which assumed pandemic proportions during the latter half of the last century, have shown no indication of slowing their growth during this first decade of the twenty first century. Challenges remain in understanding the basic mechanism of viral replication and disease pathogenesis, in clinical management of patients and in control of dengue viral transmissions. Owing to lack of any diagnostic marker and any specific clinical symptoms to identify cases that will have a severe disease outcome, early diagnosis and close monitoring with symptomatic treatment is necessary. Therefore, the aim of the study was to identify the prevalence of dengue virus among clinically suspected pediatric patients who report to the children's hospital, Chennai, Tamilnadu, India by preliminary IgM antibody detection and further confirmation with specific serotype detection by RT-PCR technique.

MATERIALS AND METHODS

Clinical Sample Collection

The study was carried out among 100 clinically suspected cases of dengue fever and clinical samples were collected from children hospitalized at Institute of child Health, Egmore, Chennai, Tamilnadu during an outbreak which started in July 2011. Ethical approval for the study was obtained from the Ethical Review Committee of the Madras Medical College, Chennai through proper channel. Demographic data were obtained using a questionnaire and relevant clinical laboratory data were recorded. Information on the duration of fever, bleeding presentation and hepatomegaly were recorded. Lower platelet count and other information used for diagnosis of dengue fever, Dengue hemorrhagic fever and Dengue shock syndrome, according to World health organization criteria WHO (1997) were also recorded. With all aseptic precautions, approximately 2.5 ml to 5 ml of venous blood was collected from patients and Sera were separated by Centrifugation at 3600 RPM for 15 minutes and stored at -70 ̊C which are used for detection of IgM antibodies and also for detecting dengue prevalence through RT-PCR.

Antibody Capture ELISA

Sera were assayed for the presence of dengue IgM antibodies using a commercial Dengue-IgM Capture ELISA test kit supplied by Arbovirus Diagnostic, National Institute of Virology, Pune, India. Among the 100 serum samples, 94 samples were analyzed to detect the dengue IgM antibodies. Based on detection by horseradish peroxidase-conjugated anti-human IgM, the assay was performed according to the manufacturer's protocol. Observances were read using an ELISA reader at a wavelength of 450nm. The color strength was immediately linked to the dengue antibody concentration in each test sample. Positive and Negative control supplied in the kit is mainly measuring the validation of the kit.

Interpretation of Results

If the OD value of samples tested exceeded OD of Negative control by a factor 4.0 (Sample OD≥ Negative OD ×4. 0), the sample was considered as positive.

DENGUE VIRUS SEROTYPING AND CONFIRMING BY RT-PCR

RNA Extraction

About 100 serum samples were tested to detect the prevalence and to confirm the serotype. Viral RNA from 140µl

was isolated directly extracted using QIAamp viral RNA extraction kit (Qiagen, India) as per the manufacturer's protocol. The RNA was eluted in 60µl of elution buffer and stored at -80°C.

RT-PCR

Dengue group-specific degenerative primers were designed targetting C-prM gene junction described by Lanciotti et al. (1992). Serotype specific primers used by Zareen et al (2011) briefly the amplified product size for specific serotypes were 411 bp, 403 bp, 453 bp, 401 bp for dengue 1, 2, 3 & 4 respectively. With these primers Reverse transcriptase Polymearse chain reaction (RT-PCR) were used for serotyping analysis of collected samples. The template was amplified by 20 µl of mixture which contains 5 µl of extracted RNA, 15 µl of PCR mixuture which contains forward and reverse primers, dNTPs, MgCl2 and Taq DNA Polymerase. The thermal profile was followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 2 minutes. The same thermal profile was followed for dengue serotype specific confirmation except the annealing was carried out at 54°C for 45 seconds in 35 cycles.

The resulting serotype specific DNA products were visualized on 1.5% agarose gel as serotype specific DNA band of Dengue 1 (411bp) and dengue 3 (453bp) by ethidium bromide staining with available markers for comparison of molecular weight. Amplified products were purified by agarose gel electrophoresis which was eluted in 30 µl of double distilled water which was used as template for sequencing reaction. Among the positive clinical samples, Dengue 1 was sequenced by adopting the protocol of Zaren et al (2011) using an ABI Sequencing machine.

STATISTICAL ANALYSIS

The numerical data obtained from the study were analyzed and the significance of difference was estimated by statistical methods. Frequency, Percentage, Mean and standard deviation are the statistical methods used in this study wherever it is applicable. Comparison between groups was done by NPAR test and Chi 'square' test as applicable. All data were analyzed by using the computer based SPSS software. A P values less than 0.05 were considered as significant where as if P value is less than 0.01 were considered as Highly significant.

RESULTS

Serological Diagnosis of Dengue Infection

A total of 100 serum samples was taken during the prevalence study of the retrospective period. Of these 100 samples only 94 serum samples were subjected for IgM captured ELISA to detect the IgM antibodies in patient's sera. Since the commercial kit contains 12 strips with 8 rows and essential of positive and negative controls, remaining samples could not be performed. Therefore, among 94 clinically suspected cases 37 (39.4%) were positive and 57 (60.6%) were negative, which is statistically significant ($P < 0.039$) by detecting NPAR test. (Table 2)

Demographic Information of Patients Enrolled in the Study

The mean age of our cohort study was in the range of 10 months to nine years. Of these, 100 pediatric samples, Dengue infections were confirmed in 37 (37%) of the patients either by serology or RT-PCR (Table 2). Among the 37 IgM positive cases, 30 (81.1%) have bleeding disorders which satisfy any one of the Dengue Hemorrhagic fever (DHF) WHO Criteria which is highly significant ($P<0.000$) and seven (18.9%) cases showed febrile illness only of dengue fever (Table 1)

Molecular Diagnosis of Dengue Infection

Enumerating the prevalence in 100 samples by molecular technique which also confirms serotype of dengue virus is promising. We could identify the virus in 12 (12%) serum samples which is statistically highly significant (P<0.000) and could not find the dengue virus in remaining 88 (88%) samples that are PCR Negative. (Table 2) Of these, 12 positive PCR samples were further processed for serotype confirmation and confirmed as 10 positive samples for dengue1 serotype which is predominant and the remaining two positive samples for dengue 3 serotype. Owing to high expenditure for sequencing, only one predominant serotype (Dengue 1) was sequenced and published in NCBI (Accession number – KC954624) and Dengue 1 is also propagated in Vero cell line for *in vitro* antiviral studies. Since the number of dengue 3 isolates are very less in number, it couldn't be sequenced and propagated. In addition, none of the IgM negative samples revealed positive for PCR

DISCUSSIONS

A dengue viral infection, which is caused by a mosquito borne virus which starts from minor troubles like fever and persist with Dengue Hemorrhagic fever, and may lead to be a major challenge to public health concern. WHO estimated occurrence of over 50 million cases of severe dengue annually with 5% lesser mortality rate. Also stated among these 90% are children who are under 15 years old. (WHO 2009) Unlike in other childhood immunizing infections, a first infection with dengue does not provide long-term protection from re-infection, as individuals are still susceptible to infection with another dengue serotypes. There is no method that can consistently determine the specific serotype to which the individuals have been exposed previously which is also difficult to diagnose. Hence for this reason, we are also unable to comment on the risk of infection among seroconverters, which would require longitudinal approaches and/ or diagnostics that are better able to differentiate between primary and secondary infections.

Owing to the importance of detecting dengue-specific serotype and percentage of infection, RT-PCR is highly recommended. The serological diagnosis is used to diagnosis the preliminary detection of IgM & IgG antibodies which generally appears 5-10 days of onset of infection. Since the antibodies appearances are very late it is difficult for early diagnosis. It is to be noted that Viral RNA could be detected even in the presence of IgM & IgG antibodies. Jose et al 2007 has also demonstrated that RT-PCR is a reliable diagnostic test in secondary dengue virus infections as well as in primary infections when serum or plasma samples collected in a febrile stage than any other test.

In this study among 100 suspected pediatric patients were processed for RT-PCR to find out the prevalence of dengue virus and also to confirm the predominant dengue serotype by type specific Polyemerase chain reaction. Of these, 12 (12%) were positive for dengue virus and 88 (88%) were negative, which is highly significant (P<0.000). Simultaneously before processing the RT-PCR technique, the clinical samples were subjected for serology (IgM ELISA) to find out the probable cases and also find out the broad dengue virus. It is very depressing to say that among the 100 serum samples, only 94 samples were processed because of the wells in commercial available kit which 96 well IgM antibody captured ELISA and also to predict the interpretation the positive and negative controls were also mandatory to perform. So, among 94 sample 37 (39.4%) were positive and 57 (60.6%) were negative, which is significant (p<0.03). However, none of the negative IgM samples are positive by PCR technique which is highly desirable. Hence, before performing RT-PCR technique for diagnosing dengue fever, IgM ELISA could be performed which is inexpensive.

Anita chakravarti et al (2006) stated that PCR technique as a method of choice for rapid, cost effective and

sensitive confirmatory diagnosis in the early phase of diagnosis infection when compared to IFAT (Immunofluorescent antibody technique) which is another method to find out the dengue serotype and also the authors declared two major factors underlying the improved sensitivity could be (i) the ability of RT-PCR to detect intact RNA inside the non-viable virus particles, which otherwise could not propagate in cell culture, resulting in false negative by cell culture and (ii) dissociation of antibody-virus complex while treating the serum samples with RNA extraction reagent containing Phenol which could have resulted in the detection of causative serotypes in the antibody negative otherwise it could be antibody positive results.

This retrospective study was conducted from August 2011 to October 2011 in pediatric patients in Chennai children's hospital. It is alarming that in the short period of three months 12 (12%) of dengue confirmed cases were identified. Atul Garg et al (2011) has also observed from their study that the dengue infection started appearing in August, peaked in October and slowly tapered by December. This shows that the presence of stagnant water after rainfall favors breeding of the mosquito vector resulting in an increased incidence of dengue.

Our present study demonstrates that Dengue 1 serotype (10%) was the dominant isolate followed by Dengue 3 (2%). The same results were also observed by Esam et al. They also reported Dengue serotype 1 as a predominant and followed by DENV3, DENV2 during study period 2006-2008. However, all the serotype is circulating which is reason of secondary dengue virus infection, which leads to DHF or DSS. The region of C-prM gene junction was selected for serotyping as the region is not very hyper variable and most of the mutation reported are of the silent type. Zareen et al (2011)

The sensitivity and specificity of laboratory diagnosis would increase by adopting a combination of virus detection by RT-PCR in the early acute phase of illness, along with serology on acute and convalescent blood samples. From the results of this study it is easy to conclude that the combination of RT-PCR and IgM ELISA increases the accuracy and sensitivity of dengue virus diagnosis.

CONCLUSIONS

Based on the findings of this retrospective study, we conclude that the predominant serotype was dengue 1 followed by dengue 3 serotype which are detected by IgM ELISA and RT-PCR techniques, finally confirmed 12 % of dengue cases identified in a short period of time. Since there is neither specific treatment, nor vaccines are available, the early diagnosis and vector control are the only way to control dengue viral infection and this will solve the problems of other mosquito borne diseases like Chikungunya, Japanese encephalitis, Malaria and Filaria.

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APPENDICES

Table 1: Crosstabulation on Seroprevalence of Dengue Cases with Bleeding Disorders

			Bleeding Disorders		Total	
			Yes	No		
Seroprevalence of Dengue virus	Positive	Count	30	7	37	P VALUE
		% within Seroprevalence of Dengue virus	81.1%	18.9%	100.0%	
		% within Bleeding Disorders	100.0%	10.9%	39.4%	
	Negative	Count	0	57	57	0.000 (Highly significant)
		% within Seroprevalence of Dengue virus	.0%	100.0%	100.0%	
		% within Bleeding Disorders	.0%	89.1%	60.6%	
Total		**Count**	**30**	**64**	**94**	
		% within Seroprevalence of Dengue virus	31.9%	68.1%	100.0%	
		% within Bleeding Disorders	100.0%	100.0%	100.0%	

Table 2: Prevalence of Dengue Fever by IgM ELISA and RT-PCR

Prevalence of Dengue Fever	IgM ELISA n (%)	RT-PCR n (%)
Positive	37 (39.4%) *	12 (12%) **
Negative	57 (60.6%)	88 (88%)
Total	**94**	**100**

P value of the above results is indicated as * Significant: ** Highly significant